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HIV-1 cDNA Integration: Requirement of HMG I(Y) Protein for Function of Preintegration Complexes In Vitro

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Summary

We present data indicating that a host protein is important for function of HIV-1 preintegration complexes (PICs) in vitro. PICs partially purified from infected cells were subjected to gel filtration in 600 mM KCl, which removed a factor required for integration without fully disrupting PICs. Addition of an extract from uninfected cells restored activity. Fractionation of the complementing activity yielded HMG I(Y), a nonhistone chromosomal protein important for transcriptional control and chromosomal architecture. Complementing activity could be isolated from PICs, and activity could be depleted from such fractions with an antibody against HMG I(Y). Recombinant HMG I(Y) also complemented salt-stripped complexes. The finding that a host protein is required for integration by HIV PICs parallels findings in several well-studied transposition and site-specific recombination systems.

Introduction

Viruses encode few of the functions necessary for their own replication, relying primarily on host activities for growth. In the case of HIV-1, several host factors have been identified, and the characterization of each has clarified central reactions in viral replication. For example, the discovery that CD4 was the HIV receptor (Maddon et al., 1986) suggested models for the origin of AIDS immunodeficiency, and the recent discovery that chemokine receptors serve as cofactors for fusion (Alkhatib et al., 1996; Deng et al., 1996; Doranz et al., 1996; Feng et al., 1996) clarified puzzling findings on viral tropism and resistance to infection in rare individuals (Dean et al., 1996; Liu et al., 1996). Host proteins carry out most of the reactions necessary for expression of viral genes and synthesis of viral proteins, and define several signal transduction pathways regulating HIV growth (Varmus and Brown, 1989). Here we report the finding of a host protein apparently required for the integration of viral cDNA into host DNA, a step in HIV-1 replication not previously known to require host activities.

The DNA breaking and joining reactions mediating integration of HIV-1 cDNA are well understood (Figure 1A) (Varmus and Brown, 1989; Farnet and Bushman, 1996). A linear form of the viral cDNA serves as the immediate precursor of the integrated provirus. Prior to integration, two nucleotides are removed from the 3′ end of each long terminal repeat (LTR), exposing recessed 3′ hydroxyl groups. The recessed 3′ hydroxyl groups then attack phosphodiester bonds on each target DNA strand so as to become joined to protruding 5′ ends. The points of joining on each strand of the target DNA are separated by five base pairs. The enzymes responsible for attaching the other viral cDNA strand to target DNA have not been identified but are likely contributed by host DNA repair systems. Gap repair at the host–virus DNA junctions completes the formation of an integrated provirus; the final gap repair step yields 5 bp duplications of the target DNA at the integration junctions.

The HIV-1 cDNA can be found in vivo in large nucleoprotein complexes termed "preintegration complexes" (PICs). PICs can be isolated from freshly infected cells, and such complexes can carry out integration in vitro when presented with a model target DNA (Brown et al., 1987; Ellison et al., 1990; Farnet and Haseltine, 1990; Lee and Coffin, 1990). Such reactions yield integration intermediates in which each viral 3′ end is attached to a 5′ end in target DNA; the subsequent gap repair step does not occur efficiently. Artificial repair of gaps generated by integration of HIV-1 PICs in vitro yields the expected 5 bp duplication of target DNA (Ellison et al., 1990; M. Hansen and F. D. B., unpublished data; K. Myrick and C. M. F., unpublished data).

PICs are composed in part of a viral-encoded enzyme—integrase—that carries out DNA-cutting and -joining reactions central to integration (Farnet and Haseltine, 1991). Purified integrase protein is capable of removing two nucleotides from 3′ ends of model LTR DNAs (Katzman et al., 1989; Roth et al., 1989; Sherman and Fyfe, 1990; Bushman and Craigie, 1991) and joining the resulting recessed 3′ hydroxyl to target DNA in vitro (Bushman et al., 1990; Craigie et al., 1990; Katz et al., 1990). Purified HIV-1 integrase alone usually carries out a partial reaction in vitro in which only one LTR substrate becomes covalently attached to target DNA (Bushman and Craigie, 1991). Both integration in vivo and integration by PICs in vitro involve the coordinated joining of two viral DNA ends (Figure 1A). Reactions with PICs also differ from reactions with purified integrase in several further respects, such as in the response to small molecule inhibitors (Farnet et al., 1996).

In addition to integrase and the HIV-1 cDNA, PICs have been reported to contain the viral matrix (MA), nucleocapsid (NC), reverse transcriptase (RT), and VPR proteins (Bukrinsky et al., 1993; Gallay et al., 1995). Although these proteins are important for the synthesis of viral cDNA (RT and NC) or subcellular sorting of HIV PICs (MA and VPR), none of the proteins are known to be important for integration itself. The cDNA in PICs exists in a folded state, with the viral ends held near one another and proteins bound at the ends (M. Miller and F. D. B., submitted). Whether further proteins are present in PICs is unknown.

In the course of developing methods to purify HIV-1 PICs, we obtained evidence that a factor important for integration in vitro was removed upon gel filtration of PICs in the presence of high salt concentrations. Activity could be restored by addition of protein extracts from

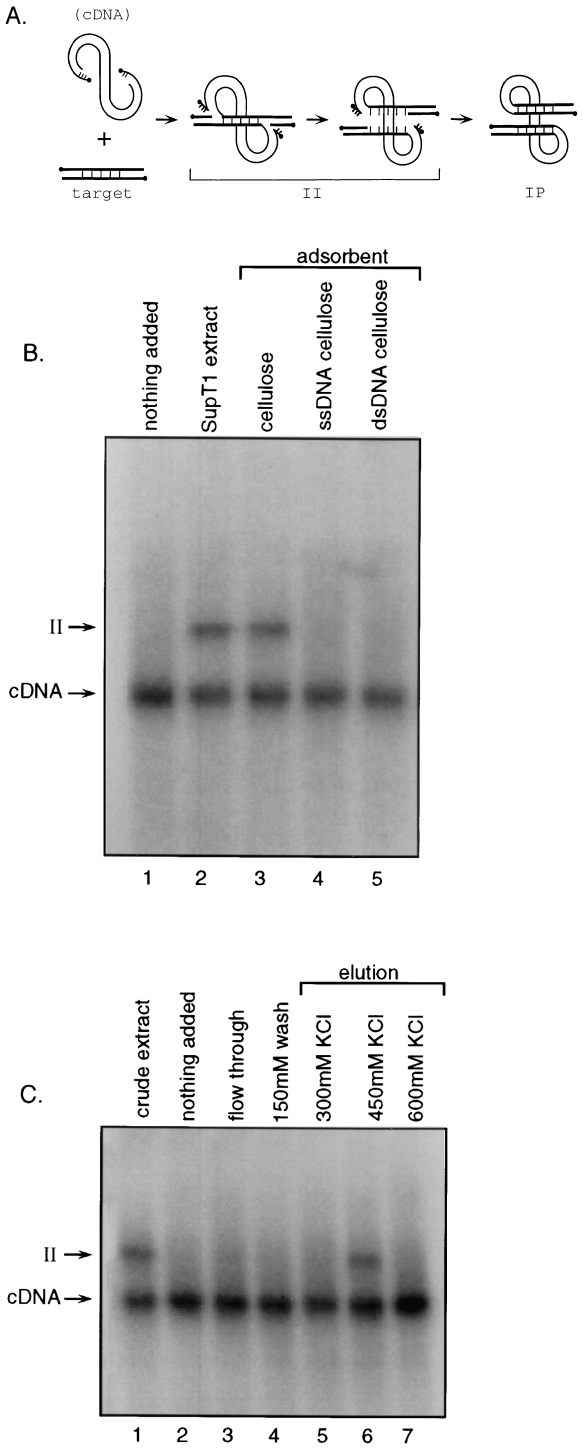

Figure 1. Integration Complementing Activity of a Host Cell Factor and Evidence for DNA Binding

(A) Inferred pathway of HIV cDNA integration in vivo. The HIV cDNA is shown by the thin lines, the target DNA by the thick lines. Balls indicate DNA 5′ ends. "II" denotes integration intermediate; "IP" denotes integration product. The five base pairs of host DNA duplicated owing to integration are shown as "rungs" in the ladder.

(B) The complementing activity binds DNA. Salt-stripped PICs were incubated with buffer alone (lane 1), untreated SupT1 extract (lane 2), or extracts depleted with the indicated materials (lanes 3–5) before lowering the salt and adding target DNA. Reaction products were analyzed by Southern blotting; "cDNA" indicates the unreacted viral DNA, "II" indicates the integration intermediate illustrated in Figure 1A. This intermediate is known to be produced in reactions with PICs.

(C) Elution of complementing activity from ssDNA cellulose. Lane 1: salt-stripped PICs reconstituted with SupT1 extract. Lane 2: unreconstituted PICs. Lane 3: PICs reconstituted with the ssDNA cellulose flow-through fraction. Lanes 4–7: salt-stripped PICs reconstituted with fractions eluted at the indicated concentrations of salt. Reactions were carried out and analyzed as in Figure 1B.

uninfected human SupT1 cells. Here we report the isolation and characterization of the complementing activity.

Results

Integration Complementing Activity in Extracts of Uninfected Cells

In the course of purifying PICs from cells infected with HIV-1, we observed that fractions containing PICs prepared by differential precipitation with ammonium sulfate often lost activity. However, remixing fractions precipitated at different concentrations of salt restored some activity. Since host factors have been found to be important in several well-characterized recombination systems (Berg and Howe, 1989; Lee and Craigie, 1994; Nash, 1996), we investigated the question of whether the complementing activity removed by high salt was derived from the host cell.

HIV-1 PICs were partially purified and then stripped by incubation in high concentrations of salt. First, PICs were precipitated in low ionic strength buffer (75 mM KCl), a step shown previously to remove at least 95% of the total protein without loss of PICs or integration activity (Farnet et al., 1996). The resulting pellets containing PICs were resuspended in buffer with 600 mM KCl and spun through CL4B gel filtration columns, which have an excluded molecular weight of 2×10^7 kDa (such complexes are referred to hereafter as "salt-stripped PICs"). The ionic strength was then lowered to 150 mM KCl. Salt-stripped PICs were not active for integration after addition of a target DNA (Figure 1B, lane 1). However, PICs subjected to gel filtration in 150 mM KCl retained activity (data not shown).

Addition of a crude extract from uninfected SupT1 cells to PICs in 600 mM KCl restored activity when the salt was lowered to 150 mM KCl and target DNA added (Figure 1B, lane 2). Integration activity returned to the previous level, in which more than half of the viral cDNA present in PICs became integrated into target DNA. Reaction products made with reconstituted PICs were characterized by cleavage with restriction enzymes (unpublished data). These data were fully consistent with the view that such reactions yielded the expected integration intermediate (marked "II" in Figure 1A and on the autoradiagrams). These data indicated that extracts of uninfected cells could provide an activity that replaced that activity lost by salt-stripping of PICs. This assay was then used to monitor fractionation steps during purification of the complementing activity.

Purification of the Complementing Activity Yielded HMG I(Y) Protein

Since some of our initial observations could be explained if the complementing activity bound to DNA, DNA cellulose was tested as a fractionation material. Double-stranded (ds) DNA cellulose, single-stranded (ss) DNA cellulose, or cellulose alone was added to complementing extracts from uninfected SupT1 cells. After incubation, the cellulose was removed by centrifugation. Complementation by the remaining supernatants was assayed by addition to salt-stripped PICs. No integration activity was present in salt-stripped PICs reconstituted with fractions depleted by dsDNA cellulose (Figure 1B, lane 5) or ssDNA cellulose (Figure 1B, lane 4). Fractions depleted with cellulose alone were able to restore activity (Figure 1B, lane 3).

Proteins bound to ssDNA cellulose were then eluted at different salt concentrations and fractions were tested for complementing activity (Figure 1C). A fraction eluting at 450 mM KCl was capable of restoring integration activity (Figure 1C, lane 6). These data supported the idea that the complementing activity could bind DNA.

The high mobility group (HMG) proteins, a class of small DNA-binding proteins extractable from chromatin with 0.35 M NaCl or 5% perchloric acid, have been reported to be able to substitute in vitro for required host factors in prokaryotic recombination systems (Paull et al., 1994; Segall et al., 1994), and thus represented candidates for the integration complementing activity. Human HMG proteins were isolated by extracting SupT1 cells with 5% perchloric acid (Sanders and Johns, 1974) and tested for the ability to complement salt-stripped PICs. The HMG fraction did possess some complementing activity, though it could not restore the level of integration to that seen with SupT1 extract (Figure 2A, lane 10). The HMG fraction also contained histone H1, which was found to inhibit integration, accounting at least in part for the inefficient complementation (data not shown).

The HMG fraction was then applied to a Mono S ion exchange column and eluted with a KCl concentration gradient. Fractions were analyzed for complementing activity (Figure 2A) and for protein content by PAGE, followed by staining with Coomassie brilliant blue (Figure 2B). Fraction 20 displayed clear complementing activity (Figure 2A, lane 6) and contained a single prominent band (Figure 2B, lane 10).

The size and fractionation profile of the species in fraction 20 were consistent with those expected for HMG I(Y) protein. HMG I and HMG Y proteins are encoded by the same gene; the two differ by an internal deletion of 11 amino acids due to alternative RNA splicing (Disney et al., 1989). The two proteins cofractionate under many conditions and are referred to together as HMG I(Y). The observed and calculated molecular weights differ, since the very high charge density of HMG I(Y) causes it to migrate unusually slowly in the SDS-PAGE system used here.

To confirm that fraction 20 contained HMG I(Y) protein, Mono S fractions were blotted to a membrane and probed with an anti-HMG I(Y) antibody. A peak of reactive material was seen in fraction 20 at the molecular weight expected of HMG I(Y) (Figure 2C, lane 9). Less reactive material was seen in flanking fractions. No reactive material was seen in other fractions from the gradient.

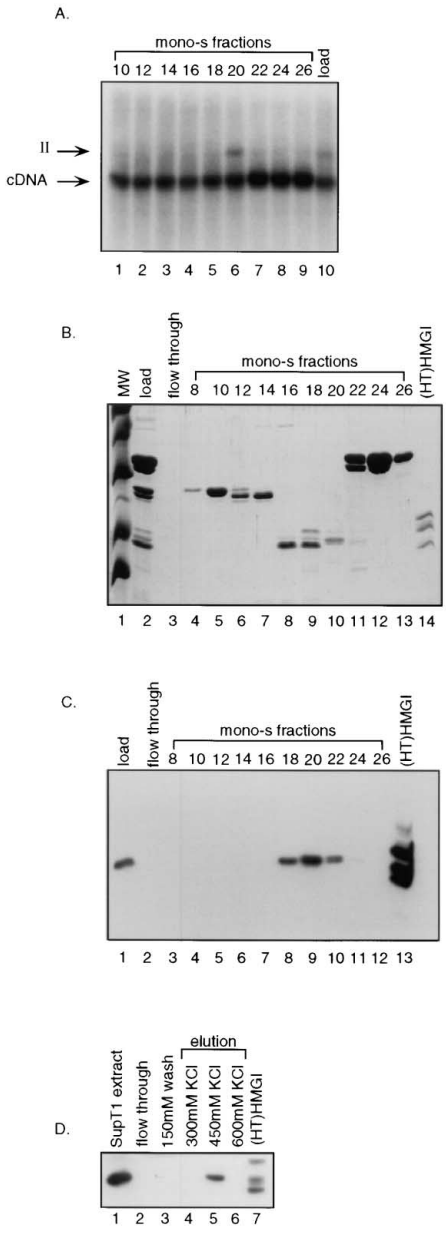

Figure 2. Purification of Complementing Activity Yields HMG I(Y)

(A) Analysis of the activity of acid extracted proteins and fractionation by Mono S chromatography. Total HMG proteins from SupT1 cells were loaded onto a Mono S column and eluted with a gradient of KCl (lanes 1–9). The initial load is in lane 10. Fractions were tested for the ability to restore activity to salt-stripped PICs and analyzed by Southern blotting as described in the Experimental Procedures.

(B) Analysis of Mono S fractions by SDS-PAGE. The lane marked (HT) HMG I contains a hexa-histidine tagged HMG I protein purified after overexpression in bacteria. The series of bands corresponds to truncations of the carboxylterminus that take place in bacteria.

(C) Western blot analysis of Mono S fractions. Fractions as in (B) were blotted to a nitrocellulose membrane and probed with an α-HMG I antibody.

(D) Western blot analysis of fractions eluted after binding to ssDNA cellulose as described in Figure 1C. Blots were probed with an α-HMG I antibody.

The fractions obtained earlier by elution from ssDNA cellulose (Figure 1C) were then assayed for their HMG I(Y) content by Western blotting (Figure 2D). A blot probed with an anti-HMG I(Y) antibody revealed a band of the size expected for HMG I(Y) in the fraction eluted with 450 mM KCl (Figure 2D, lane 5), i.e., the fraction that displayed complementing activity (Figure 1C, lane 6).

Thus, two methods for fractionating the complementing activity from SupT1 cells—digitonin permeabilization followed by DNA cellulose fractionation and 5% perchloric acid extraction followed by Mono S chromatography—each yielded HMG I(Y). Abundant HMG I(Y) was not found in any fractions that failed to display complementing activity.

HMG I(Y) Is Present in HIV-1 PICs

The above data indicate that HMG I(Y) is capable of supplying complementing activity but do not address the question of whether HMG I(Y) supplies this activity in PICs as originally isolated. An extreme alternative would be that salt-stripping of HIV-1 PICs removes some required factor other than HMG I(Y). According to this idea, addition of HMG I(Y) might restore activity but not restore the original composition of PICs.

To address this possibility, PICs were first analyzed to determine whether HMG I(Y) was present. PICs were spun through gel filtration columns and the presence of HMG I(Y) was assessed on Western blots probed with an anti-HMG I(Y) antibody. A fraction containing PICs subjected to gel filtration in the presence of 150 mM KCl displayed a reactive protein at the molecular weight expected for HMG I(Y) (Figure 3A, lane 2). A fraction containing PICs subjected to gel filtration in the presence of 600 mM KCl, in contrast, did not contain detectable HMG I(Y) (Figure 3A, lane 3). An extract from uninfected SupT1 cells prepared in a similar fashion and subjected to gel filtration in 150 mM KCl did not contain HMG I(Y) (Figure 3A, lane 4). Recovery of PICs after gel filtration was confirmed by Southern blot analysis of DNA purified from the fractions analyzed (data not shown). These data indicate that HMG I(Y) is associated with HIV-1 PICs and removed by gel filtration in 600 mM KCl.

Parallel studies of viral proteins (data not shown) revealed that integrase protein, MA, and RT were present in fractions after gel filtration in 150 mM KCl and remained associated after gel filtration in 600 mM KCl. NC and CA proteins, in contrast, were undetectable in fractions after gel filtration in 150 mM KCl, indicating that large amounts of these proteins were not tightly associated.

Antibodies against HMG I(Y) Deplete Complementing Activity Eluted from PICs

To test whether HMG I(Y) protein in PICs is important for function, complexes were treated with 600 mM KCl

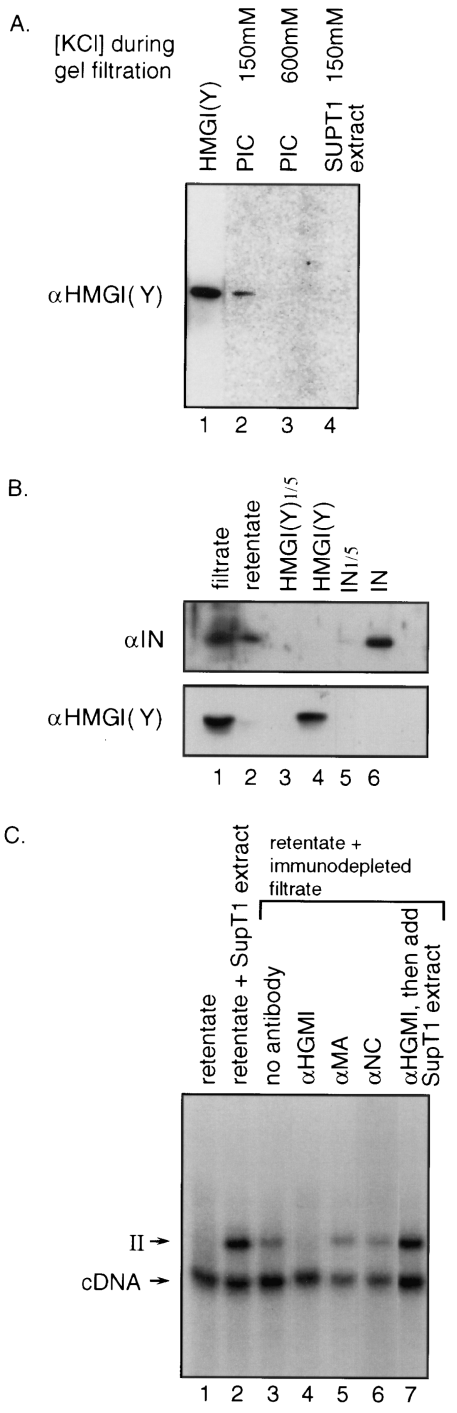

Figure 3. HMG I(Y) Is Present in Partially Purified PICs, and Integration Complementing Activity from PICs Can Be Depleted with an α-HMG I(Y) Antibody

(A) Western blot detection of HMG I(Y) in PIC fractions after gel filtration. Lane 1: HMG I(Y) protein standard. Lane 2: PICs resuspended in 150 mM KCl were purified by gel filtration in CL-4B spin columns equilibrated in 150 mM KCl. Lane 3: PICs were resuspended in buffer containing 600 mM KCl and purified by gel filtration in 600 mM KCl. Lane 4: extract from uninfected SupT1 cells was subjected to gel filtration in 150 mM KCl.

(B) Western blot analysis of protein composition in filtrate and retentate fractions after Centricon-100 ultrafiltration. Lanes 1 and 2 contain 15 μl of the filtrate and retentate fractions from Centricon-100 ultrafiltration, respectively; lanes 3–6 contain integrase and HMG I(Y) protein standards.

(C) Southern blot analysis of reactions containing complementing activity depleted with different antibodies. The antibodies used for depletion are marked above the gel over lanes 3–6. Lane 7 was reconstituted with filtrate depleted with the α-HMG I(Y) antibody, then SupT1 extract was added.

and the complementing activity released was then recovered and analyzed. PICs were partially purified by precipitation in reduced ionic strength buffer, then resuspended in buffer containing 600 mM KCl. Samples were then fractionated by ultrafiltration in Centricon-100 units. Components of molecular weight less than 100 pass through such filters, while components of higher molecular weight, such as PICs, are retained. PICs in 4 ml were reduced in volume to 40 μl by ultrafiltration. PICs were retained by the ultrafiltration membrane. Complexes were washed once in high salt buffer and, when tested, activity was found to be completely abolished (Figure 3C, lane 1). Reconstitution with SupT1 extract (Figure 3C, lane 2) or the filtrate fraction (Figure 3C, lane 3) restored integration activity.

The protein compositions of the retained PICs and filtrate fractions were then investigated by Western blotting. HIV integrase protein partitioned about equally in the filtrate and retentate fractions (compare Figure 3B, lanes 1 and 2, α-integrase). It is not clear whether the free integrase was derived from the active PICs studied or contaminating subviral material. A band of the size expected for HMG I(Y), in contrast, was detected in the filtrate fraction (Figure 3B, lane 1, α-HMG I[Y]). Only trace amounts of HMG I(Y) were seen in the retained salt-stripped PICs (Figure 3B, lane 2). Evidently, HMG I(Y) was removed from the PIC fraction by ultrafiltration in 600 mM KCl, while much of the integrase protein remained with PICs.

Several antibodies were then assayed for the ability to deplete the filtrate-complementing activity released from PICs. Antibodies against HMG I(Y), HIV-1 MA, or HIV-1 NC were added to filtrate fractions, and bound proteins recovered by binding to protein A sepharose beads. Antibody-depleted filtrate fractions were then used to reconstitute salt-stripped PICs and integration assayed. Antisera against HMG I(Y) removed the complementing activity (Figure 3C, lane 4). Depletion of the filtrate with antibodies against HIV-1 MA or HIV-1 NC failed to remove the complementing activity (Figure 3C, lanes 5 and 6). The α-HMG I(Y) antibody preparation was not nonspecifically inhibitory to integration, since complexes treated with the α-HMG I(Y) antibody-depleted filtrate could be restored to activity by subsequent addition of SupT1 extract (Figure 3C, lane 7).

The data in Figure 3 support the view that HMG I(Y) is present in PICs as initially isolated and required for function in this setting.

Complementation with Purified Proteins

To strengthen the evidence that HMG I(Y) was the complementing activity, HMG I protein was overexpressed in E. coli, purified, and tested for complementing activity. HMG I was purified either as a fusion protein containing a hexahistidine tag (Thanos and Maniatis, 1992), or as the authentic polypeptide purified by acid extraction and Mono S chromatography. Purified HMG I was added to salt-stripped HIV-1 PICs, and the formation of integration products monitored. In the presence of HMG I purified by either means, abundant integration products were formed (Figure 4, lanes 3–8). About 20%–50% of reconstituted complexes carried out integration reactions, approaching, though not equaling, the original level of 60%–80% integration. Very high concentrations appeared to be slightly inhibitory (Figure 4, lane 3). Purified recombinant HMG Y was also able to reconstitute integration activity to the same level as HMG I (data not shown).

Many other candidate proteins were tested for complementing activity. All of the HMG proteins present in SupT1 cells were tested, as shown in Figure 2. In addition, purified human HMG-1 and HMG-2 were also tested separately at several concentrations and found to be unable to supply complementing activity (Figure 4, lanes 15–20; unfortunately, for historical reasons unrelated HMG proteins are named HMG-1 and HMG I). Several additional DNA-binding and DNA-bending proteins from diverse sources were also tested (Table 1). In all cases, assays were carried out at a minimum of 3 concentrations across at least a 100-fold concentration range.

Some reconstituting activity was detected with high levels of HIV-1 NC protein (Figure 4, lanes 9–11). Twenty micrograms of NC protein per reaction could promote integration of about 10% of the viral cDNA. Two and a half micrograms of HMG I(Y), in contrast, could promote integration of about 50% of the cDNA. The antibody depletion experiments in Figure 4 indicate that HMG I(Y) provides the predominant complementing activity present in PICs, and compositional studies indicate that NC is not present in PICs as isolated, arguing against a major role for NC. Detergent-lysed HIV-1 virions, which contain abundant NC, were unable to supply complementing activity (Figure 4, lanes 12–14). HIV-1 MA and integrase proteins were also unable to supply complementing activity at the concentrations tested (Figure 4, lanes 24–26, and data not shown).

Discussion

Here we present data indicating that the high mobility group protein HMG I(Y) is required for function of HIV-1 preintegration complexes (PICs) isolated from HIV-1-infected cells. We found initially that integration activity was lost from PICs following treatment with 600 mM KCl. Integration activity could be restored, however, by addition of extracts from uninfected SupT1 cells, indicating that a host activity might be required. Purification of the complementing activity yielded HMG I(Y) protein. HMG I and HMG Y proteins overexpressed in E. coli and purified from that source were also sufficient to restore integration activity to salt-stripped PICs. Analysis of protein composition by Western blotting revealed that HMG I(Y) was present in PICs, but depleted from PICs by high salt treatment. Complementing activity could be obtained from preparations of PICs, and the activity in such fractions specifically was removed by treatment with an α-HMG I(Y) antibody. Purified HMG I(Y) alone was not sufficient to carry out integration when mixed with purified HIV-1 cDNA (data not shown). These data support a model in which HMG I(Y) is required as an accessory factor for the function of HIV-1 PICs.

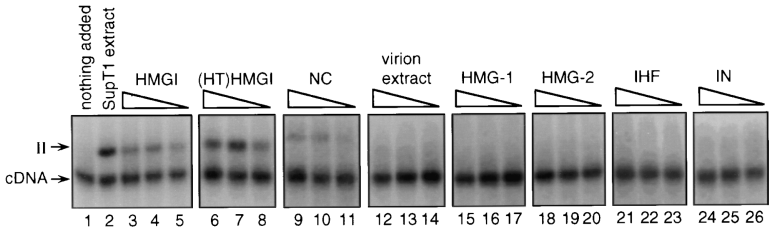

Figure 4. Complementation with Purified Proteins

Salt-stripped PICs were reconstituted with the indicated proteins and activity analyzed by Southern blotting. Amounts of proteins added: lane 3–5: 25, 5, and 1 μg HMG I; lane 6–8: 2.5, 0.8, and 0.3 μg (HT) HMG I; lane 9–11: 20, 4, and 0.8 μg HIV-1 NC; lane 12: 10 μl virion extract; lanes 13 and 14, 1:5 and 1:25 dilutions of virion extract; lanes 15–17: 12.5, 1.25, and 0.125 μg of HMG-1; lanes 18–20: 12.5, 1.25, and 0.125 μg of HMG-2; lanes 21–23: 25, 2.5, and 0.25 μg of IHF; lanes 24–26: 20, 2, or 0.2 μg of HIV-1 IN protein. Reaction volumes were 100–110 μl.

Possible Roles of HMG I(Y) in HIV-1 cDNA Integration

Our data allow us to identify at least one step in the integration pathway that specifically requires HMG I(Y). The covalent chemistry of integration involves two steps: cleavage of the viral cDNA ends to expose recessed 3′ hydroxyls and covalent joining of the viral cDNA ends to phosphodiesters in the target DNA. We find that the majority of the viral cDNA ends are cleaved in PICs as initially isolated (M. Miller and F. D. B., submitted). The finding that salt-stripped PICs with precleaved ends are inactive indicates that HMG I(Y) is important at least for the covalent strand transfer step.

As yet, the mechanism by which HMG I(Y) promotes integration is unclear, though several models can be adduced. One model posits that HMG I(Y) acts by binding to integrase, perhaps acting as an allosteric effector. However, attempts to demonstrate binding between HMG I(Y) and purified HIV-1 integrase in vitro so far have been unsuccessful (unpublished data), failing to strengthen this hypothesis. HMG I(Y) might bind other proteins in the PIC; no data are yet available that bear on this possibility. It seems unlikely that HMG I(Y) acts by binding to the target DNA, since addition of HMG I(Y) to salt-stripped PICs after addition of target fails to complement (unpublished data). Since target DNA is present in great excess over HIV cDNA, it seems likely that target DNA also serves as a competitor in this setting, trapping HMG I(Y) and blocking binding to PICs. If HMG I(Y) acted by binding to target DNA, no such inhibition would be expected.

Given the available data, it seems simplest to propose that HMG I(Y) acts by binding to the HIV cDNA. HMG I(Y) binds DNA as a monomer (Reeves and Nissen, 1990; Maher and Nathans, 1996) at A/T rich sequences, particularly sites containing at least five sequential A or T residues, though binding is usually not highly sequence specific (Strauss and Varshavsky, 1984; Elton et al., 1987; Reeves et al., 1987). Potential binding sites for HMG I(Y) are present in the sequences of the HIV LTRs (bases 11–15, 22–27, 249–253, 426–431, 439–443, 607–611, and 622–626; numbers refer to the left LTR of pNL4–3). Perhaps binding of HMG I(Y) alters the conformation of the HIV cDNA so as to promote formation or function of the catalytically active complex.

One simple role for HMG I(Y) might be to bring the two ends of the viral cDNA together in the active complex. The ends must be brought into proximity normally, since the points of joining of the two ends in target DNA are always spaced by 5 bp (Vincent et al., 1990; Vink et

Table 1. Summary of Complementing Activity Present in Different Protein Fractions

Protein or Compound	Complementing Activity?	Source
HMG I(Y)	+	SupT1 cells
HMG I	+	E. coli expression
(HT)HMG I	+	E. coli expression
HIV-1 NC	+/−	E. coli expression
Serum albumin	−	Bovine
Total Histones	−	Calf thymus
Histone H1	−	Calf thymus
poly-lysine	−	
protamine sulfate	−	Salmon sperm
T4 gene 32 protein	−	E. coli expression
RNase A	−	Bovine pancreas
RNase T1	−	Aspergillus oryzae
IHF	−	E. coli expression
HU	−	E. coli
SSB	−	E. coli expression
Uracil DNA glycosylase	−	Human
glyceraldehyde 3-phosphate dehydrogenase	−	Human
RPA	−	Human
LEF DNA-binding domain	−	E. coli expression
HMG-1	−	Human
HMG-2	−	Human
HIV-1 IN	−	E. coli expression
HIV-1 MA	−	E. coli expression
HIV-1 virion extract	−	Molt IIIB producer cells

al., 1990; Stevens and Griffith, 1996). A protein bridge is implicated in linking the cDNA ends, since cleavage of the viral cDNA internally with a restriction enzyme does not disrupt correct coupled joining (M. Miller and F. D. B., submitted). However, one prediction of the idea that HMG I(Y) bridges the cDNA ends is not met. We find that salt-stripped PICs do not display uncoordinated joining of single cDNA ends to target DNA. Such reactions would yield Y-shaped DNA molecules, and these are not detected either as new product DNA species on gels or as a depletion of the unreacted cDNA. If removal of HMG I(Y) does disrupt the association of the two cDNA ends, this must be accompanied by reversible inactivation of integrase.

Other Proposals for Auxiliary DNA-Binding Proteins Involved in Retroviral Integration

A host protein has also been proposed to influence MLV integration, in this case preventing MLV PICs from using their own cDNA as an integration target (Lee and Craigie, 1994). The active host protein fraction has not been fully characterized, and it is unknown whether the factor studied is present in normal MLV PICs. It may be of interest to compare the function of this possible host component with that of HMG I(Y).

In several previous studies, reactions in vitro containing purified integrase protein have been reported to be stimulated or inhibited by addition of other DNA-binding proteins (Fujiwara and Craigie, 1989; Bushman and Craigie, 1990; Bushman et al., 1990; Katz et al., 1990; Kalpana et al., 1994; Aiyar et al., 1996). The biological significance of these effects is unclear, since many different proteins, including prokaryotic proteins, have been shown to provide the generally modest effects observed. We find that HMG I(Y) can also stimulate function of purified integrase slightly (unpublished data), but whether this stimulation reflects normal function in vivo is unclear. It will be interesting to see if the proteins reported to influence activity of purified integrase can be found in PICs and shown to be important for function in this context.

Roles of Host DNA-Binding Proteins in Transposition and Site-Specific Recombination

The finding that a host DNA-binding protein is important for function of HIV PICs parallels previous findings in several site-specific recombination and transposition systems. Normal integrative recombination by lambda-phage integrase, for example, requires the participation of the E. coli protein IHF, which binds to the viral DNA and bends it into the active conformation (for review, see Nash, 1996). Mu-phage transposition requires the participation of E. coli Hu protein, which binds to the left end of Mu DNA and helps bring monomers of MuA transposase into the active alignment (see Lavoie et al., 1996, and references therein). E. coli Fis protein, another DNA-bending protein, is important for formation of DNA-inversion complexes by Gin and Hin invertases (for review, see Merker et al., 1993). In each case, the active recombination complex is composed of several monomers of the recombination enzyme precisely organized with the host proteins on substrate DNA. HMG I(Y) resembles IHF, Hu, and Fis in that it is a small host DNA-binding protein that, when bound, alters the conformation of DNA (Thanos and Maniatis, 1992; Reeves and Nissen, 1993; Lehming et al., 1994; Falvo et al., 1995; Nissen and Reeves, 1995; Thanos and Maniatis, 1995; Reeves and Wolffe, 1996), possibly mediating its role in HIV integration.

Purified HIV-1 integrase protein is active in vitro in the absence of HMG I(Y) under certain conditions, again potentially paralleling results of studies of prokaryotic recombination systems. Conditions in vitro have been identified in which lambda-phage integrase, MuA transposase, and Gin and Hin invertases each display catalytic activity in the absence of the host-encoded cofactor (Johnson et al., 1986; Kanaar et al., 1986; Craigie and Mizuuchi, 1987; Nash, 1990; Savilahti et al., 1995). These reactions without host factors generally fail to mimic recombination in vivo faithfully, displaying reduced efficiency, nonbiological reaction requirements, or yielding nonbiological reactions products. Similarly, for the case of HIV, reactions in vitro with purified integrase mainly produce partial reaction products that mimic events at one end of the viral cDNA only. Evidently, the full context of the PIC is required to produce authentic products efficiently, and this setting confers a requirement for HMG I(Y).

HMG I(Y) as a Possible Sensor for Regulatory Inputs

In some of the prokaryotic recombination complexes, the requirement for host cofactors may have evolved to link recombination activity with regulatory inputs, and the same may hold for HMG I(Y) in HIV. Fis protein, for example, varies in abundance at least 70-fold, depending on the growth state of E. coli cells (Thompson et al., 1987). HMG I(Y) varies in abundance, depending on growth state, and furthermore has been reported to be posttranslationally modified by phosphorylation, adenylation, acetylation, methylation, and glycosylation (Elton and Reeves, 1986). Phosphorylation of HMG I(Y) has been reported to reduce the DNA-binding affinity (Nissen et al., 1991; Reeves et al., 1991). Possible influences of these posttranslational modifications on integration can now be investigated using the PIC system.

The subcellular distribution of HMG I(Y) may also be significant for HIV replication. HMG I(Y) is not detectable in virions (unpublished data), so PICs must acquire HMG I(Y) after infection of target cells. HMG I(Y) has been reported previously to be concentrated in the nucleus (Disney et al., 1989), so it is possible that in vivo integration is restricted to this compartment. This may be advantageous, since, in the cytoplasm, PICs might try to use the only available DNA, their own cDNA, as an integration target. Lack of HMG I(Y) in the cytoplasm may render the integration machinery inactive, potentially preventing HIV from carrying out suicidal autointegration.

Experimental Procedures

PIC Preparation

Infections were initiated by cocultivating human SupT1 cells (2×10^6 cells/ml, grown in RPMI1640 medium containing 10% fetal calf

serum) with phorbol 12-myristate 13-acetate-stimulated MoltIIIB cells at a ratio of approximately 50:1. Cell extracts were prepared 5 hr after initiation of the coculture, as previously described (Farnet and Haseltine, 1990). Crude extracts, containing approximately 0.5 ng/ml viral cDNA and 2 mg/ml protein, were frozen in 2 ml aliquots at −70°C.

Salt-Stripping of PICs

PICs in 2 ml of crude SupT1 extract were pelleted following precipitation in low ionic strength buffer (Farnet et al., 1996). Pelleted PICs were resuspended in 510 μl of buffer T containing 600 mM KCl (buffer T600; buffer T is 20 mM Tris [pH 8.0], 5 mM $MgCl_2$, 1 mM DTT, 10 units/ml aprotinin). To separate salt-stripped factors from preintegration complexes, the resuspended PICs were divided into 3 aliquots of 170 μl and centrifuged over 3 Sepharose CL-4B spin columns (1.5 ml bed volume each) equilibrated in buffer T600. Eluate fractions contained salt-stripped PICs corresponding to a concentration of approximately 2 ng viral cDNA per ml.

For the experiments described in Figure 3A, PICs from 2 ml of extract were resuspended in 170 μl of buffer T containing 600 mM KCl, and centrifuged over a CL-4B column equilibrated in the same buffer. Non-salt-extracted PICs were prepared by resuspending PICs in buffer T containing 150 mM KCl and centrifuging over a CL-4B column equilibrated in the same buffer. Control uninfected SupT1 extracts were treated at low ionic strength, and the pelleted material was resuspended in buffer T containing 150 mM KCl and centrifuged over a CL-4B column in the same buffer. Ten microliter aliquots of the CL-4B eluates were analyzed for viral cDNA and integration activity. Protein present in 150 μl aliquots was precipitated by acetone and analyzed on 15% SDS-polyacrylamide gels, followed by Western blotting by standard methods (Harlow and Lane, 1988) using an HMG I(Y) antiserum.

PIC Assays

Twenty-five microliters of salt-stripped PICs prepared by CL-4B gel filtration were incubated with 10 μl of buffer or protein fractions at room temperature for 10 min. Buffer T containing 30% glycerol was then added at a volume sufficient to reduce the monovalent cation concentration to 150 mM, and the samples were incubated at room temperature for an additional 10 min. Three microliters of linearized ΦX174 RF DNA (at 0.3 mg/ml in 150 mM KCl, 10 mM Tris HCl [pH 8.0], 5 mM $MgCl_2$, 1 mM DTT) was then added, and the reactions were incubated at 37°C for 1 hr. Reaction products were purified and analyzed by Southern blotting, as previously described (Farnet and Haseltine, 1990). Integration efficiency was determined by PhosphorImager analysis of Southern blots.

Ultrafiltration of PICs in High Salt

For the experiment described in Figure 3B, PICs from 2 ml of extract were precipitated by incubation at low ionic strength and resuspended in 525 μl of buffer T600. Five hundred microliters of the sample was centrifuged for 1 hr at 1000 × g at room temperature in a Centricon-100 ultrafiltration unit (Amicon), generating a 40 μl retentate fraction and a 450 μl filtrate fraction. Two microliters of retentate was incubated with 25 μl of buffer T600 or 25 μl of the filtrate fraction before diluting with buffer T containing 30% (w/v) glycerol to reduce the KCl concentration to 150 mM. After a 10 min incubation, target DNA was added and the reaction incubated for 1 hr at 37°C. For the experiment described in Figure 3C, PICs from 6 ml of extract were resuspended in 510 μl of buffer T600 and subjected to ultrafiltration in the same manner. The retentate (40 μl) was diluted with 500 μl fresh buffer T600 and subjected to ultrafiltration a second time to remove residual salt-stripped factors. Filtrate from the first ultrafiltration was diluted with 3 volumes of buffer T to reduce the KCl concentration to 150 mM. For immunodepletion of the filtrate, 5 μl of the indicated antiserum was incubated with 200 μl of filtrate for 2 hr at 4°C. Immune complexes were precipitated by adding 25 μl of protein A-sepharose CL-4B beads (Pharmacia) for 1 hr and centrifuging briefly.

Uninfected SupT1 Extracts

Uninfected SupT1 extracts were prepared using the same extraction buffer used to isolate PICs from infected cells. Cells were washed once in buffer K (20 mM HEPES [pH 7.5], 150 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 10 units/ml aprotinin) and permeabilized by incubating for 10 min in 15 ml of buffer K containing 0.025% digitonin. Cell debris was pelleted by centrifugation at 1000 × g for 3 min. The supernatant was clarified by centrifugation at 8000 × g for 3 min, adjusted to 10% (w/v) glycerol and 0.5 mM AEBSF, and stored at −70°C.

For the fractionation of uninfected SupT1 extracts, extract from 5×10^8 cells was loaded onto an ssDNA cellulose column (1 ml bed volume, 3.6 mg DNA/gram cellulose, Sigma) equilibrated in buffer K containing 10% (w/v) glycerol. Proteins were eluted with a step gradient consisting of 300 mM KCl, 450 mM KCl, and 600 mM KCl in buffer K containing 10% (w/v) glycerol. One milliliter fractions were collected at each salt concentration, dialyzed against buffer K containing 10% (w/v) glycerol and 0.5 mM AEBSF, and stored at −70°C.

For the cellulose depletion experiments, 15 mg of cellulose (Sigmacel type 50, Sigma), ssDNA cellulose (3.6 mg DNA/gram cellulose, Sigma), or dsDNA cellulose (3 mg DNA/gram cellulose, Sigma) was added to 250 μl of uninfected SupT1 extract and incubated at 4°C for 30 min with constant mixing. Celluloses were pelleted by centrifugation at 10^4 × g for 1 min, and the supernatants collected and stored at 4°C until used.

For the experiments in Figures 3 and 4, extracts from uninfected SupT1 cells were prepared by a modification of the procedure of Wobbe et al. (1985). SupT1 cells (5×10^8) were washed two times in cold PBS and once in hypotonic buffer (20 mM HEPES [pH 7.5], 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 10 units/ml aprotinin, 0.5 mM AEBSF). All subsequent steps were performed at 4°C. Cells were resuspended in 3 ml hypotonic buffer and incubated for 10 min. Fifteen microliters of 5% digitonin (w/v in DMSO) was added, and incubation continued for 20 min before adding 125 μl of 5 M NaCl. After a 10 min incubation, the cell lysate was centrifuged at 10^5 × g for 30 min. The supernatant fraction was dialyzed against 20 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10% (w/v) glycerol, 10 units/ml aprotinin, 0.5 mM AEBSF, clarified by centrifugation at 10^5 × g for 30 min, and stored in aliquots at −70°C.

Purification of HMG Proteins from SupT1 Cells

HMG proteins were prepared from SupT1 cells by a modification of the perchloric acid extraction procedure of Sanders and Johns (1974). SupT1 cells (2×10^9) were washed two times in cold PBS. All subsequent steps were performed at 4°C. The cell pellet was resuspended in 20 ml 5% perchloric acid and homogenized with 20 strokes in a dounce homogenizer. The homogenate was incubated on ice for 30 min before spinning at 2000 × g for 15 min. The pellet was reextracted with 10 ml of 5% perchloric acid, and the two supernatants pooled. The supernatant fraction was filtered through a 0.45-micron filter and acidified by adding 0.03 vol of concentrated HCl. Proteins were precipitated by adding 6.2 vol of acetone and pelleted by centrifugation at 2000 × g for 30 min in a swinging bucket rotor. The HMG protein pellet was washed twice with cold acetone, dried, resuspended in 3 ml of buffer A containing 10 mM NaCl (buffer A is 10 mM Tris HCl [pH 7.5], 1 mM DTT, 10% (w/v) glycerol), and filtered through a 0.45-micron filter. Proteins were fractionated by FPLC using a Mono S column (HR 5/5, 1 ml bed volume, Pharmacia). The HMG proteins were loaded onto a column equilibrated in buffer A containing 10 mM KCl and eluted with a 0.01–1 M gradient of KCl in buffer A at a flow rate of 1 ml/min. Twenty-seven fractions (0.5 ml) were collected, pooled in groups of two, and dialyzed against 20 mM HEPES (pH 7.5), 50 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 10% (w/v) glycerol, 10 units/ml aprotinin, 0.5 mM AEBSF. Protein in the fractions was detected by electrophoresis on 15% SDS-polyacrylamide gels followed by Coomassie blue staining.

Purification of HMG I(Y) from E. coli Cells after Overexpression

HMG I(Y) was purified from E. coli cells by two methods. In one case, a modified version of HMG I containing an amino-terminal hexahistidine tag was purified on chelating sepharose charged with nickel, essentially as described (Thanos and Maniatis, 1995). This yielded a preparation containing three bands, each of which reacted

with an α-HMG I(Y) antibody. Evidently, the two shorter peptides were truncated near the carboxyl-terminus. In the second method, a plasmid (pET7CIY) directing expression of authentic HMG I(Y) (Reeves and Nissen, 1993) was used. E. coli strain BL21/DE3 containing pET7CIY was grown to mid-log in Terrific Broth (Sambrook et al., 1989) and induced by adding IPTG to a concentration of 1 mM. After 2.5 hr, cells were harvested, washed in 10 mM Tris (pH 8), and lysed by adding lysozyme to 1 mg/ml, followed by incubation for 30 min on ice and sonication. The lysate was extracted by adding perchloric acid to 5% (vol/vol) and dounce homogenized with 20 strokes. The lysate was centrifuged for 20 min at 3000 rpm in a Sorval RC 3B rotor, and the supernatant filtered through a 0.45-micron filter. The filtrate was acidified by addition of 0.03 vol of concentrated HCl, and precipitated by addition of 6.2 vol of acetone. After centrifugation as above, the pellet was washed twice with cold acetone, dried, and resuspended in 3 ml of buffer A with 50 mM NaCl. The resuspended acetone pellet was then applied to a Mono S column (Pharmacia) and eluted with a 0.05–1 M gradient of NaCl in buffer A. Peak fractions contained a single prominent band. Analysis of the protein in this fraction by mass spectrometry and amino-terminal sequencing indicated that it had the molecular weight expected for HMG I(Y) after removal of the amino-terminal methionine.

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